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鹤顶兰胚囊发育过程中Ca^{2+}分布的超微细胞化学定位*

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摘要：用焦锑酸盐沉淀法对鹤顶兰（*Phaius tankervilliae*）胚囊发育过程中的Ca^{2+}状态进行超微细胞化学定位。观察结果发现：功能大孢子时期，珠孔端的胚囊壁上开始出现小颗粒的Ca^{2+}沉淀，但功能大孢子细胞内未见明显的Ca^{2+}标记；四核胚囊时期胚囊壁上的Ca^{2+}沉淀明显增多，液泡膜上有Ca^{2+}沉淀出现，珠孔处的Ca^{2+}沉淀颗粒较大；成熟胚囊时期，胚囊壁上的Ca^{2+}沉淀进一步增多，且胚囊内Ca^{2+}分布明显增多，且极性明显，珠孔端助细胞、卵细胞比合点端反足细胞有更多的Ca^{2+}沉淀。鹤顶兰成熟胚囊内Ca^{2+}积累的来源有：（1）在胚囊成熟前主要由珠被细胞、珠细胞通过胞间连丝向胚囊运输；（2）以沉淀有大量Ca^{2+}的小泡形式跨过胚囊壁进入胚囊。

关键词：鹤顶兰；胚囊；超微细胞化学定位；焦锑酸盐沉淀法；钙

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Ultracytochemical Localization of Calcium during Embryo Sac Development in *Phaius tankervilliae* (Orchidaceae)

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Abstract: Ultracytochemical localization of calcium in embryo sac of *Phaius tankervilliae* (Aiton) Bl. was carried out using potassium pyroantimonate precipitation method. Results of Ca^{2+} distribution during embryo sac development are as following: (1) Megasporocyte stage: There is no Ca^{2+} precipitation in the megasporocyte, nucellar cell and micropyle; (2) Functional megaspore stage: Small particles of Ca^{2+} precipitates appear on the embryo sac wall at the micropylar end, but no apparent Ca^{2+} can be found in the functional megaspore; (3) 4-nucleate stage: There are significant increase of Ca^{2+} precipitation on the embryo sac wall and large grains of the precipitates in the micropyle, while a little starts to appear on the membrane of vacuole; (4) 8-nucleate embryo sac stage: Ca^{2+} precipitation continuously increases on the embryo sac wall. Distribution of Ca^{2+} in embryo sac shows strong polarity. More Ca^{2+} precipitations are observed in the synergids and the egg cell than the antipodal cells. Accumulation of Ca^{2+} precipitation is achieved by (1) transferring Ca^{2+} to embryo sac through the plasmodesmata between the nucellar cells, which mainly occurred at the chalaza end before the embryo matured; (2) forming large amounts of small bubbles of Ca^{2+} precipitation, which can cross embryo sac wall to enter embryo sac.

Key words: *Phaius tankervilliae*; Embryo sac; Ultracytochemistry; Potassium antimonite technique; Ca^{2+}

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The distribution and evolution of Ca^{2+} has profound influence on the process of metabolism and development (Antoine *et al.*, 2000). The effect of Ca^{2+} on the sexual reproduction of plant appears to be one of the important factors in the study on plant reproductive biology (Ge *et al.*, 2007). Previous studies demonstrated that Ca^{2+} signals are involved in the whole process from the beginning of pollen reaching the stigma to the end of double fertilization (Yang *et al.*, 2002). Most studies have focused on pollen development, pollen germination and growth *in vitro* , the distribution in mature embryo sac, as well as the effects during double fertilization. High content of Ca^{2+} was commonly observed in the synergids cells (Tian and Russell 1997; Yu *et al.*, 1998, 1999), and in the region of pollen germination and growth. In addition, Ca^{2+} is often found in the apoplast (Yu *et al.*, 1998; Mao *et al.*, 1992; Zhang *et al.*, 1995, 1997), indicating that Ca^{2+} plays a key role in the process of pollen tubes entering embryo sac and sperm cells releasing. However, how does the Ca^{2+} distribution change before embryo sac maturation? For instance, megasporogenesis and embryo sac development, to what extent are they related to the distribution of Ca^{2+} within embryo sac (Qiu *et al.*, 2005; 2008). There have been quite few reports on these questions so far. To further explore effects of Ca^{2+} on sexual reproduction, we have conducted ultracytochemical localization of calcium in embryo sac of *Phaius tankervilliae* (Aiton) Bl. We present the results of a systematic investigation on the distribution of Ca^{2+} and distribution changes during megasporogenesis and embryo sac early development. And the ways that Ca^{2+} accumulates in embryo sac are also discussed in this paper, which may have far-reaching significance on sexual reproduction.

Materials and methods

Materials *Phaius tankervilliae* (Aiton) Bl. plants were grown in South China Botanical Garden. Flowers were pollinated at anthesis. Ovules were dissected at different stages during ovary development.

Methods Samples were prepared using potassium pyroantimonate precipitation method. Ovules were fixed in 2% glutaraldehyde in 0.1 mol·L^{-1} potassium phosphate buffer (PBS, pH7.6) containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ for 4 h at room temperature, washed in five changes of 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ in 0.1 mol·L^{-1} PBS buffer (20 min each) and postfixed in 1% OsO_4 for 16 h at 4℃ in 0.1 mol·L^{-1} PBS buffer containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$. Control was prepared with following modifications: 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ was omitted from solutions during processing, and washed in five changes of 0.1 mol·L^{-1} PBS buffer without antimonite. Specimens were dehydrated in graded ethanol, displaced by epoxy dimethylmethane and embedded in Epon812 resin. Thin sections at thickness of 80—90 nm were cut using LK-S ultramicrotome, stained with 2% uranyl acetate for 60—90 min, then observed with Japanese TEM-1010 transmission electron microscope at 90 kV and took photograph.

Results

The embryo sac belongs to a Pangianum type in its development, which is consistent with previous reports (Ye *et al.*, 1996; Tung *et al.*, 2000). In this section, the distribution of Ca^{2+} precipitates and its evolution during the development of embryo sac are described.

Megasporocyte stage

The megasporocyte, which originates from archesporial cell, possesses large volume, thick wall and distinct nucleus. Before the meiotic prophase, the megasporocyte is highly polarized and the nucleus tends to locate at the micropylar end. The density of cytoplasmic contents is higher at the chalazal end which contains a large amount of organelles, such as mitochondrion, dictyosome, endoplasmic reticulum, etc. In contrast, the micropylar end has less cytoplasm. At this stage, no Ca^{2+} precipitates can be found in the megasporocyte, nucellar cell, embryo sac wall and micropyle. (Plate Ⅰ: 1)

Functional megaspore stage

The megasporocyte undergoes meiosis, and gives rise to a tetrad. The cell at chalazal end grows further and forms functional megaspore, while the other three cells close to the micropylar end degenerate (Plate Ⅰ: 2). The functional megaspore contains numerous small vacuoles, in which some flocs begin to appear. The cytoplasmic electron density is low. Small Ca^{2+} precipitations start to accumulate on the embryo sac wall near the micropylar end, but not at the interior of functional megaspore. Some Ca^{2+} precipitations are observed at the micropyle, and in the nuclei of nucellar cells. (Plate Ⅰ: 2, white arrowheads).

4-nucleate embryo sac stage

The functional megaspore undergoes mitotic division and gives rise to a 2-nucleate embryo sac. Right after that, the second mitotic division occurs, leading to 4-nucleate embryo sac (Plate Ⅰ: 3A, 3B). Increases of Ca^{2+} precipitations are evident on the embryo sac wall, and numerous small grains of Ca^{2+} precipitations begin to appear on the embryo sac walls that are close to the chalazal end. At the chalazal end, continuous distribution of Ca^{2+} precipitations exit between the embryo sac wall and the cytoplasm of nucellar cells (Plate Ⅱ: 4). The content of Ca^{2+} at outer integument is very much at the stage of four-nucleate embryo sac (Plate Ⅱ: 5, 6). There is a Ca^{2+} stream which starts from the outer integument to embryo sac wall, in a zigzag way across the inner integument (Plate Ⅱ: 7). A large number of granular precipitations are observed in the vacuoles of 4-nucleate embryo sac, degenerated nucellar cells and inner integument, especially on the cell wall of outer integument. Continuous distribution of Ca^{2+} can also be seen at the apoplast system of outer integument, inner integument, nucellar cells and embryo wall (Plate Ⅱ: 7).

8-nucleate embryo sac

Through the third mitotic division, eight-nucleate embryo sac replaces the four-nucleate ES, some Ca^{2+} precipitations are observed at the visual nucleus and cytoplasm (Plate Ⅱ: 8). There is a further increase of Ca^{2+} precipitation on embryo sac wall and significant Ca^{2+} precipitation at the micropyle (Plate Ⅱ: 9). The mature embryo sac of *Phaius tankervilliae* (Aiton) Bl. contains seven cells. Distribution of Ca^{2+} precipitations shows strong polarity within mature embryo sac, relative low Ca^{2+} precipitation is present at the chalazal end, spotty precipitations in antipodal cells and a few small dots on the vacuolar membrane of central cell (Plate Ⅲ: 10), large precipitation grains remain at the cell wall of inner degenerated integument (Plate Ⅲ: 11), small bubbles containing a large amount of Ca^{2+} precipitation can be seen near the wall inside embryo sac (Plate Ⅲ: 12). Before fertilization, a synergid cell distinctively has degenerated (Plate Ⅲ: 13). Ca^{2+} precipitations in egg cell (Plate Ⅲ: 14) and synergids (Plate Ⅲ: 15) are abundant, Small bubbles, which exhibit aggregates of precipitation dots throughout its membrane, are occasionally found within embryo sac.

Discussion

Ca^{2+} play very important roles on the structure and physiological function of plant cell, especially in double fertilization of angiosperm, but it is unfeasible for cells to have high content of Ca^{2+} for long time, owing to the calcium toxicity which is associated with the reaction between Ca^{2+} and PO_4^{3-} (Rasmussen, 1981; Rasmussen and Barrett, 1984). At megasporocyte stage, our observation result is similar to Lettuce that there is no Ca^{2+} precipitates can be found (Qiu *et al.*, 2005). There is a polar distribution of Ca^{2+} precipitation in mature embryo sac in *Phaius tankervilliae*. The polar distribution of Ca^{2+} precipitation is generally consistent with the observations from *Brassica napus*, rice and cotton (Yu *et al.*, 1998; 1999; Zhang *et al.*, 1997). So far, few works on how the Ca^{2+} accumulate at the embryo sac.

In this study, we employing an ideal experiment material, *Phaius tankervilliae*, about 45 days from megasporocyte to mature embryo sac (Li *et al.*, 2006). The distribution of Ca^{2+} and its evolution during megasporogenesis and early embryo sac development were revealed systematically with transmission electron microscopy.

The transfer of Ca^{2+} from outer integument to nucellar cells and embryo sac is responsible for the accumulation of Ca^{2+} in the mature embryo sac. According to the study on the formation and development of embryo sac wall in rice (Liu *et al.*, 1997), two ways of nutrition conveyance before the 8-nucleate embryo sac formats 7 cells are assumed. In one way, nutrition passes through the cytoplasm of cells linked by plasmodesmata (the symplast), which mainly occurred on embryo sac at the chalazal end, in the other way, nutrition penetrates into embryo sac through the embryo sac wall and the spaces between cells (the apoplast). After the formation of 7 cells, nutrition penetrating into embryo sac through the apoplast becomes the major way. Ultracytochemical localization of calcium was carried out in the degenerating nucellus and endosperm cell of rice post flowering (Wei, 2002), suggesting that the calcium was transported to embryo sac through two patterns: one way is that calcium was transported by some little bubbles containing aggregates of Ca^{2+} precipitations; the other way is that a Ca^{2+} stream starts from the degenerated vacuole of nucellar cells in a zigzag way across embryo sac wall. We observed the similar phenomena during embryo sac development of *Phaius tankervilliae*. At the stage of 4-nucleate embryo sac, remarkable Ca^{2+} precipitations are found at the plasmodesmata between the nucellar cells and embryo sac in the chalazal end (Plate Ⅱ: 4), and more abundant Ca^{2+} precipitations were found in outer integument than inner integument (Plate Ⅱ: 5, 7). As the embryo sac matured, the degenerated inner integument has much Ca^{2+} precipitation (Plate Ⅲ: 11), small bubbles containing a large amount of Ca^{2+} precipitation can be seen near the wall inside embryo sac (Plate Ⅲ: 12).

Based on these observations, we propose that Ca^{2+} accumulates in mature embryo sac in two ways: before embryo sac maturation, Ca^{2+} is mainly transported through plasmodesmata from integument to nucellar cells, then to embryo sac; at the stage of mature embryo sac, Ca^{2+} migrates through embryo sac wall in the form of small bubble containing aggregates of Ca^{2+} precipitation.

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Explanations of Plates

A. Antipodal cell; DS. Degenerated synergid cell; DM. Degenerated megaspore; E. Egg cell; EW. Embryo sac wall; FM. Functional megaspore; II. Inner integument; M. Megasporocyte; Mit. Mitochondrion; N. Nucleus; NC. nucellar cell; OI. Outer integument; S. Synergid cell; V. Vacuole

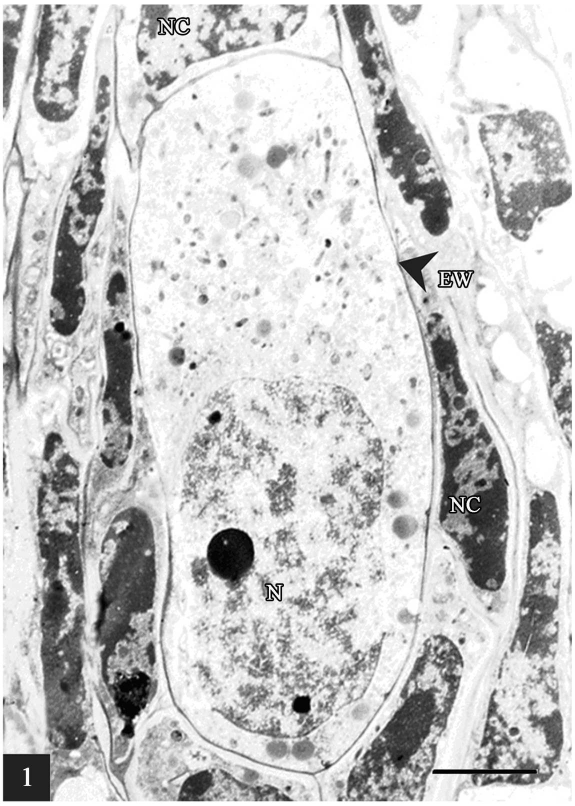
Plate Ⅰ: 1-3B　Micropyle downward in figures and note the Ca^{2+} deposits with white arrowheads　1. Development of a megasporocyte, the large nucleus is located at the micropylar end, no Ca^{2+} precipitations have been observed. bar=7 μm; 2. Functional megaspore showing some Ca^{2+} precipitations in the micropyle, nucellar cells and the nuclei of functional megaspore (white arrowhead). bar=2 μm; 3A. 4-nucleate embryo sac. bar=5 μm; 3B. 4-nucleate embryo sac (another grid), two nuclei are located at the chalazal end, a large number of granular Ca^{2+} precipitations are showing at the vacuole. bar=5 μm

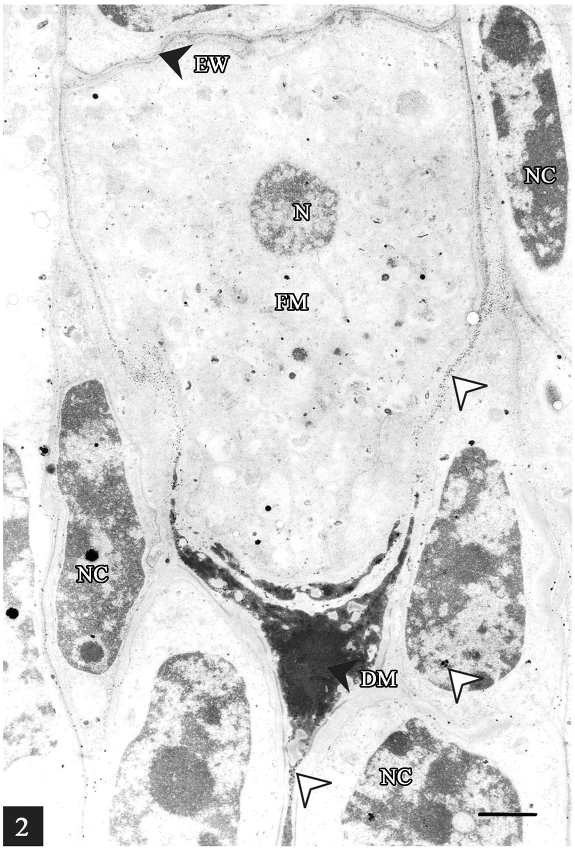
Plate Ⅱ: 4-9　Micropyle downward in figures and note the Ca^{2+} deposits with white arrowheads　4. Higher magnification of square at 3B showing Ca^{2+} distribution at the embryo sac wall and nucellar cells at the chalazal end. bar=500 nm; 5. Abundant Ca^{2+} at outer integument of 4-nucleate embryo sac. bar=1 μm; 6. Integument of 8-nucleate embryo sac, Ca^{2+} precipitation at outer integument is lower than that at the stage of 4-nucleate embryo sac. bar=2 μm; 7. Integument of 4-nucleate embryo sac showing Ca^{2+} precipitations distribution at outer integument, inner integument and embryo sac wall. bar=2 μm; 8. 8-nucleate embryo sac, the synergids and egg cell are located at the micropylar end. bar=7 μm; 9. Higher magnification of square at the micropyle of 8, Ca^{2+} precipitations are significant. bar=500 nm

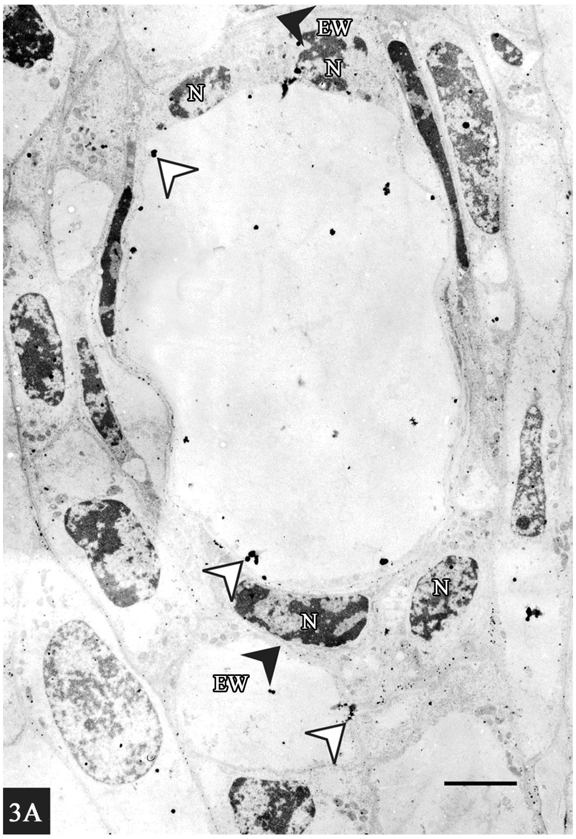
Plate Ⅲ: 10-15　Micropyle downward in figures and note the Ca^{2+} deposits with white arrowheads　10. Mature embryo sac, three antipodal cells are located at the chalazal end, the nuclei of egg cell is at another grid, showing relative low content of Ca^{2+} precipitation at antipodal cells and some small grains at the vacuole membrane of central cell. bar=1 μm; 11. Higher magnification of square a at 10, large Ca^{2+} precipitations grains remain at cell wall of inner degenerated integument. bar=1 μm; 12. Higher magnification of square b at 10, small bubble containing significant Ca^{2+} in mature embryo sac. bar=200 nm; 13. Mature embryo sac before fertilization, a synergid cell distinctively has degenerated. bar=5 μm; 14. Higher magnification of egg cell at 13, showing Ca^{2+} precipitation grains at the cytoplasm and nucleus of egg cell. bar=200 nm; 15. Higher magnification of square at 13, abundant Ca^{2+} precipitation at the degenerated synergid cell. bar=500 nm

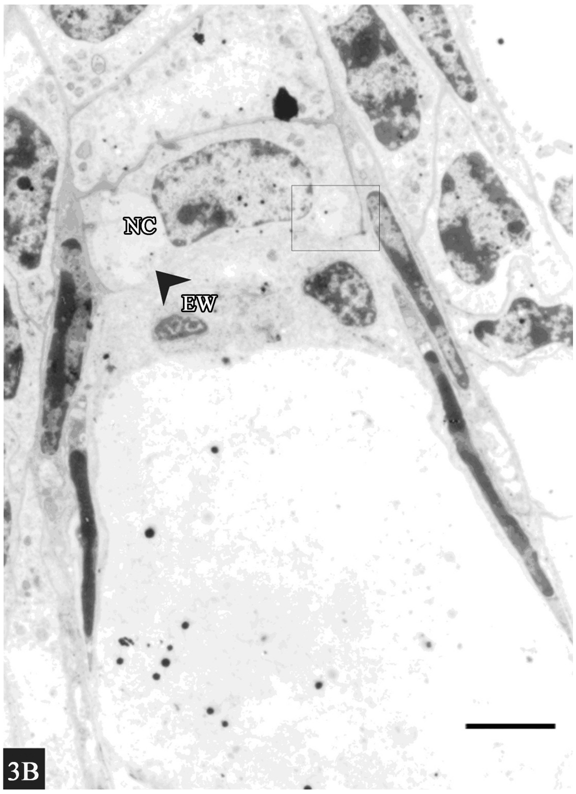
李冬妹等：图版Ⅰ

LI Dong-Mei *et al*.: Plate Ⅰ



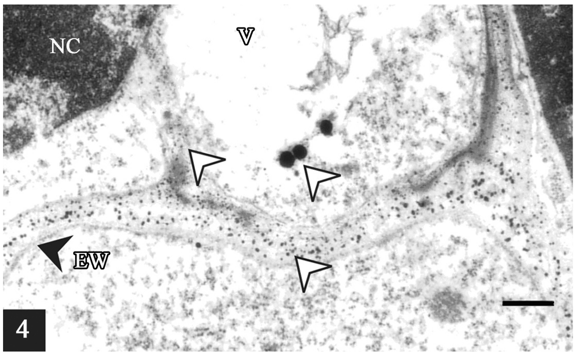






李冬妹等：图版Ⅱ

LI Dong-Mei *et al*.：Plate Ⅱ



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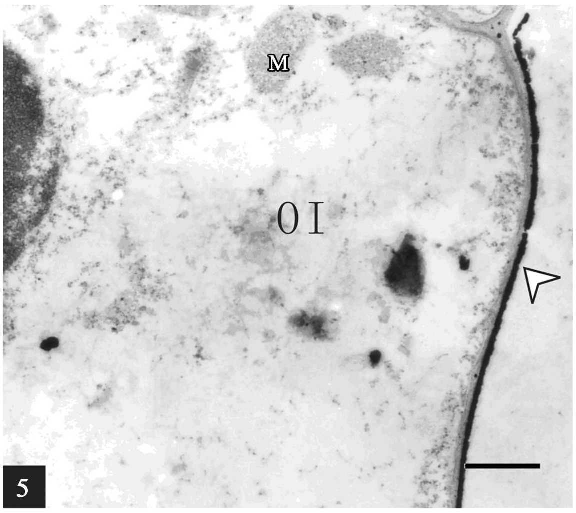

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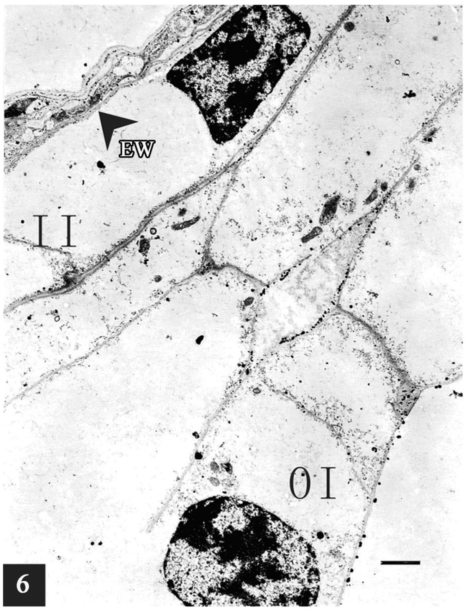

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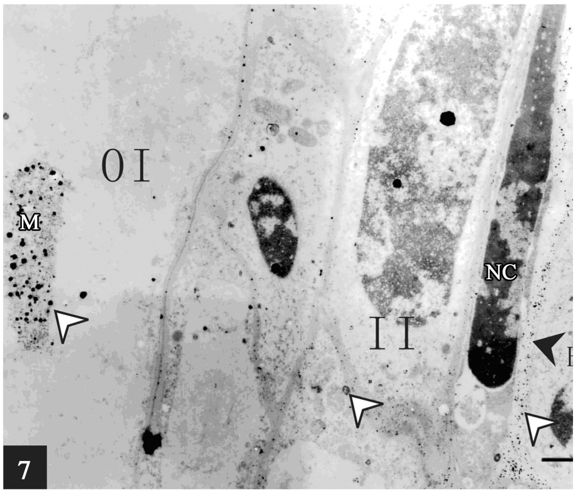

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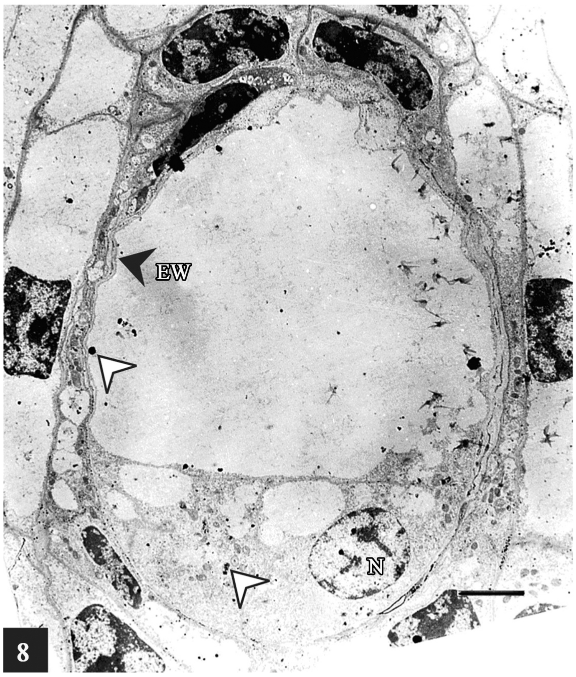

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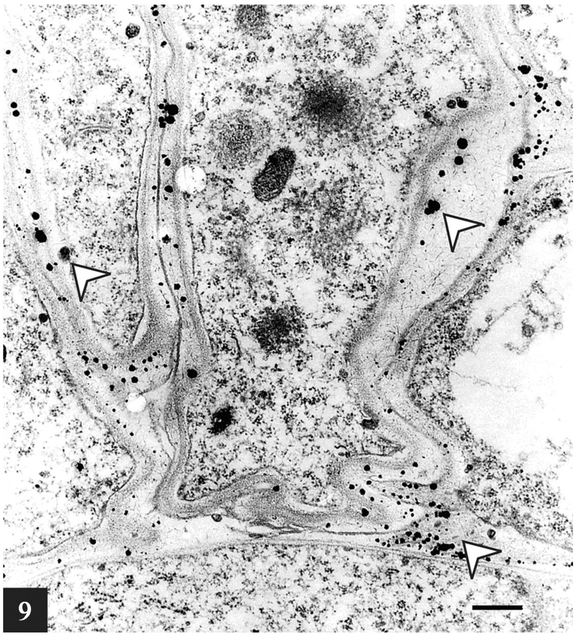

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李冬妹等：图版Ⅲ LI Dong-Mei *et al*.: Plate Ⅲ